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MEDIA AND METHODS OF CULTURE ADAPTED TO THE REANIMATION AND MULTIPLICATION "IN VITRO" OF MYCOBACTERIUM TUBERCULOSIS OF REDUCED VITALITY, EPHEMERAL VIABILITY OR IN A STATE OF QUIESCENCE

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The hierarchy of values in the isolation and identification of Mycobacterium tuberculosis (M. t.) in order of increasing sensitivity is as follows:

a) Microscopic examinations;

b) Culture on favorable media;

c) Inoculation into laboratory animals, the guinea pig in particular.

Since the arrival of the antibacillary agents we see a progressive inversion of the preceding rule.

1. In many cases, the sensitivity of the guinea pig is less than that of a culture. This possibility is very frequent, we know, with INH-resistant bacilli.

2. In other cases, whose number increases each year, while simple microscopic examinations show bacilli and sometimes in a considerable number, it is not rare to find nothing with culture and guinea pig inoculation.

These failures, with bacilli which are morphologically intact for microscopic examination, do not seem to conform and are in discordance with the clinical and epidemiological data.

On the clinical plane, with the persistence of active anatomo-radiological lesions being the rule, the pathogenic role of these bacilli for the man who harbors and eliminates them, cannot be doubted.

On the epidemiological plane, some observations are troubling:

1. While most of the contaminating agents spread mono or poly-resistant bacilli, it is exceptional that strains, isolated from patients, recently affected and not yet treated, are, at first, resistant.

2. Even though chronic tuberculars eliminate mostly INH-resistant, and thus avirulent, bacilli, their entourage is exposed to risks of certain contamination.

3. Statistically, in spite of the reduction of tuberculosis mortality in considerable proportions, tuberculosis morbidity is still high (1).

All of these questions remain unanswered and the acceptance by biologists of the possibility of bacilli called "visible but non-viable" makes the explication and interpretation of these facts still more difficult.

These biological, clinical and epidemiological problems are distinct in appearance but directly dependent on the bacillus. They seem, however, to be joined together:

1. In the fundamental heterogenicity of bacillary populations.

2. In biological (metabolic, enzymatic, etc.) modifications caused in bacilli by the systematic use of antibiotics.

3. In the regression of the vitality and virulence of most strains of M.t., a regression which does not need to be exclusively and integrally attributed to antibacillary agents but all the measures which are part of the antituberculosis fight.

These ideas, strictly concerning the M.t. in vitro culture, raise and justify a certain number of questions:

1. How can we pretend to assure, in vitro, the ideal physiological conditions, the needs for salts and mineral catalysts, the vitamins and organic substances which are indispensable for all and each of the elements which compose a bacillary population?

2. Would the vital needs be the same for the bacilli spontaneously expulsed from the organism (expectoration, urine, fistulised ganglion) and those taken by force by puncture, biopsy or surgical excision?

Is it rational to envision identical conditions of life:

a) For bacilli living in the lung in free communication with the bronchials, and those imprisoned in encysted loci?

b) For bacilli in a multiplying phase and those in a "quiescent state?"

3. How can we effectively supply the multiple and complex needs of bacilli which have been exposed during varying times and in various degrees to the harmful effects of chemotherapy?

If, as we should, we take these different factors into consideration (the cultural failures being there to remind us), we are forced to admit that a single polyvalent cultural medium will not be simple or easy to produce.

The bacteriologist, searching for a satisfactory answer to the questions above, must apply himself:

1. To assure the perennity of bacilli which transferred in vitro have a reduced vitality or an ephemeral viability. Example: these microcolonies which abort on the spot or painfully arrive at a macroscopic state after a prolonged incubation, and which are little viable because they cannot be transferred.

2. To reanimate the bacilli, which persist and live slowly in the organism: the bacilli in a quiescent state (2, 3) responsible for relapses, also little or non-viable in vitro.

3. To make morphologically intact bacilli which are disturbed in their metabolism and biosynthesis aptitudes to the point of being incapable of utilizing the nutritive elements present in our cultural media, live outside the organism.

In that respect the hypothesis, according to which these bacilli would find substances whose nature is difficult to determine in the human body, presently seems well founded.

Also, before reviewing the methods to remedy these difficulties, we have tried to analyze and study two large orders of factors:

1. Extrinsic factors (4);

2. Intrinsic factors (5) of M.t., responsible for cultural failures on standard media.

Parallelly, we have tried to explain the mechanism of going into quiescence for M.t. and we have experimentally demonstrated the possibility of reanimating these bacilli in vitro (3, 5).

From all of our research these facts stand out:

a) The harmful effect of extrinsic factors;

b) The importance of intrinsic factors;

c) The necessity of not forgetting the fragility and vulnerability of M.t. in regard to physical, chemical and medicinal agents.

These considerations seem indispensable to us to place the problem of the bacilli called "visible and non-viable" in its correct proportions.

"INTERSTIMULATION" PHENOMENON

One of the particularities of bacilli with reduced vitality and viability is the "interstimulation" phenomenon.

By this term, we mean the mutual reenforcing of the vitality of bacilli in groups in relation to their survival difficulties in a dispersed unit state.

With bacilli having normal vitality, in progressive "dilutions," actually more and more extensive dispersions, the number of colonies decreases in a regular manner, with profit, for the "dilution" which follows in relation to that which precedes. Example: for 500 colonies at 10^{-4} mg., we will often have 60 to 80 colonies at 10^{-5} and 8 to 12 colonies at 10^{-6} mg.

This is due to the fact that groups of 3 to 10 bacilli each give birth to a colony, in the same way an isolated unit and groups of 2 or 3 bacilli give the same result.

The addition of a spreading agent (Tween 80) in the bacillary suspensions makes the decreasing curve more regular; however, a slight advantage persists for the intermediary "dilutions," which disappears for the high "dilutions" where the risk in the division of units is unforseeable.

For bacilli with a diminished vitality and in identical inoculation techniques, there is an inverse phenomenon. Example: going from a bacillary suspension which gives 50 colonies per tube, to the following 1/10 dispersion, the success of the culture is risky or zero, compared to a constantly positive culture of a strain with normal viability.

If, in addition, the dispersion coincides with transplantation (change of media), the phenomenon becomes even more remarkable and transferring is always negative unless large bacillary concentrations are used.

The "interstimulation" or "group effect" is also manifested in some circumstances in bacilli having normal viability. Example: the success of a pellicle (veil) culture on Sauton's medium, which requires a state of intimate contiguity between the different portions of the inoculant. It actually suffices to disperse the inoculant on the surface of the media or to prevent the contiguity by very weak vibrations for the pellicle culture to fail completely.

The "interstimulation" or "group effect" also occurs in vivo, for example in the release of experimental tuberculosis in little receptive laboratory animals (rats, mice), where inoculations of small amounts of bacilli produce no effect.

Isn't there reason to establish a relation between these biological circumstances and the cases of first infections occurring in the immediate entourage of patients eliminating bacilli reputed to be avirulent or non-viable?

Consequently, during the transplanting of bacilli from their usual life medium to a very different and less favorable medium (this is always the case for a first culture), the "group effect" can be useful for bacilli with a normal vitality and absolutely indispensable for bacilli with a reduced vitality, because they support and reenforce each other.

With this line of thinking and with the goal of improving the chances of success with the first culture, we have attempted to prove the role of extemporaneous contributions of live or dead bacillary bodies to inoculation.

For this, we used either dead bacilli, or human or bovine type, or [illegible] type, killed by tyndallization or U.V. radiation and with the tuberculin (unfavorable factor for in vitro culture) removed, or either live bacilli not growing at 38° C (M. balnei type).

The smallness of the results obtained in both cases can be attributed to the destruction of enzymes during tyndallization and to enzymatic differences between M. balnei and M.t.

Practical conclusion for the first culture: as much as possible avoid dispersion of the bacilli present in a pathological product each time the viability and vitality of the bacilli in it are doubted.

CHOICE OF A FAVORABLE MEDIA FOR THE FIRST CULTURE OF *M. tuberculosis*

On a purely scientific plane one can only be satisfied with the development of one or more synthetic media which assure the in vitro culture of bacilli called normal and those called "visible but non-viable."

While waiting for this to occur through the combined research of biochemists and bacteriologists, two types of media seem presently to answer best the requirements of the M.t. first culture.

1. THE SEMI-SYNTHETIC MEDIA. -- Those of Dubos (6, 7, 8) and Youmans (9), in a liquid state assure an accelerated multiplication for M.t. It is thus normal that media of this type, solidified by the addition of agar, be used for the first culture of M.t. (10, 11). However, their use is limited by the fact that:

a) Frequent contaminations: unfortunately, the addition of penicillin to protect them has a prejudical effect on the development of bacilli with reduced vitality and viability; the same occurs with malachite green when it is added in a titer higher than 20 $\mu g/cm^3$ while in egg medium, the titer of 200 $\mu g/cm^3$ (ten times higher) does not have the same disadvantages.

b) Their lower sensitivity compared to that of the egg medium, Jensen's medium in particular, for the isolation of M.t. from pathological products (12, 13, 14).

c) Difficulties with morphological evaluation and individual study of the colonies.

2. THE SOLID EGG MEDIA. -- Since Dorset's media (15), many preparations have been proposed: Lowenstein (16), Herold (17), Petragnani (18), American Trudeau Society A.T.S. (19), Jensen (20). Of all these media the most sensitive, incontestably is Jensen's with 0.75% glycerin.

MEDIA ADAPTED TO THE FIRST CULTURE OF M. tuberculosis HAVING A REDUCED VITALITY AND VIABILITY

As early as 1950, thus before the discovery of INH, certain cultural failures from surgically extracted material led us to search for the possible causes (21).

Elsewhere, certain contaminations, either by mold or other germs have the particularity of lowering the pH of the medium, consequently yielded positive M.t. cultures while the intact tubes remained negative. Also, in some positive M.t. tubes we found a manometric depression of - 10, - 20, and - 30 cm of water while the homologous tubes with normal pressure remained negative. These two facts led us to study:

1. The role of the substances secreted by the contamination germs;

2. The influence of pH of the medium on M.t. during incubation;

3. The role of an atmosphere poor in O_2 (5, 22).

Parallelly, we tried to study the influence of some well defined chemical products, vitamins, amino acids and a range of vegetal, microbial or animal extracts or filtrates on the culture of M.t.

Our experiments these last 10 years have borne mostly on bacillary populations mostly coming directly from man, the collection strains being little demonstrative under the circumstances.

Our goal was to identify and select the products or substances favorable to the development of bacilli having a reduced vitality and viability.

The excessive slowness of our investigations was due to the fact that:

a) The culture of dysgonic bacilli, in the etymological sense of the term, often takes three or more months;

b) The products which are non-prejudicial to the development of normal bacilli and favorable for bacilli with reduced vitality, are rather rare;

c) The margin between the threshold of activity and the threshold of tolerance for each of these products and substances is very thin. This is the case for Mn, vitamins and a number of amino acids (23).

Also, we were not astonished that instead of vitamins and amino acids whose inconsistent and often contradictory effects, we preferred to use oligoelements whose role as catalysts capable of starting biosynthesis is much more interesting.

Sodium pyruvate and glutamate as well as litmus blue (Madagascar litmus) held our attention and became part of the base medium we propose because of their stimulating action on a wide range of M.t. (24).

The role of ossein in the medium will be described further on.

Our base medium always assures the in vitro development and multiplication of the large majority of bacilli with normal or slightly reduced vitality. The composition and preparation method of this medium are given on the following pages.

However, for some bacillary populations mostly composed of bacilli profoundly disturbed in their metabolism and enzymatic functions, and because of this, incapable of synthesizing in vitro, the extemporaneous addition of substances having organic vegetal (mushrooms, [illegible], gibberellin), microbial (extracts or bodies of common germs or [illegible]), or animal (organ extracts) origin are indispensable.

But the selection of substances of organic origin is still delicate and difficult, because they are subject to more variations than the well defined chemical products.

In conclusion, if we have opted for monkey organ extracts, certainly the delux solutions, but imposed by the very particular requirements of the "visible and non-viable" bacilli, it is because the organ extracts from other less precious animals -- rabbit, guinea pig, veal, etc. -- have been found to be less effective.

Table 1. -- Total composition

	Pasteur Institute T.B.C. Medium	
	Base Medium (Composition)	
	For 1 liter of eggs, yields 1,600 liters of medium	For a 100 ml. medium yield
Monopotassium phosphate	2.4 g.	0.15 g.
Magnesium sulfate	0.24 g.	0.015 g.
Magnesium citrate	0.6 g.	0.0375 g.
L Asparagine	3.6 g.	0.225 g.
Sodium Pyruvate	1.6 g.	0.1 g.
Sodium Glutamate	1.6 g.	0.1 g.
Litmus blue R.A.L.	0.4 g.	0.025 g.
Anthracite Ashes	0.16 g.	0.01 g.
Oligodynamic solution	1.6 ml.	0.1 ml.
Bidistilled glycerin	12 ml.	0.75 ml.
Potatoe-starch	16 g.	1 g.
2% Malachite Green	16 ml.	1 ml.
Distilled water	440 ml.	27.5 ml.
4%, pH 6.8 steril casein	160 ml.	10 ml.
Eggs whole	800 ml.	50 ml.
Eggs yolks	200 ml.	12.5 ml.

BO medium (base medium + 20% exuded ossein)
"song cane" Culture

Same composition as the base medium, only the ossein titer is different: use 160 ml. of 20% ossein instead of 160 ml. of 4% ossein in the base medium.

CONSTITUTING ELEMENTS

Solution of salts

(For 1 liter of eggs or 1,600 liters of medium)

Pour into a pyrex balloon flask:

Monopotassium Phosphate	2.4	g
Magnesium sulfate	0.24	g
Magnesium Citrate	0.6	g
l Asparagin	3.6	g
Sodium Pyruvate	1.6	g
Sodium Glutamate	1.6	g
Litmus blue, hydrosoluble lichens R.A.L.	0.4	g
Bidistilled glycerin	12	ml
Distilled water	440	ml

To be dissolved by heat in a boiling water bath (the color is that of an onion skin). The pH is 4.4.

Oligodynamic solution of A. Berthelot and G. Bertrand (25)
(See note)

Note: The complete original formula contains $(SO_4)_3Fe_2$, 50 g. per liter, at the head of the list, which we eliminated.

H_2O	1 000 ml
$SO_4Mn\ 7H_2O$	2 g
$SO_4Ca\ 2H_2O$	0,50 g
$Cl_2Ni\ 6H_2O$	0,05 g
$Cl_2CO\ 6H_2O$	0,05 g
$(SO_4)_2\ Th$	0,20 g
$SO_4Zn\ 7H_2O$	0,10 g
$SO_4Cu\ 5H_2O$	0,05 g
$SO_4Gl\ 4H_2O$	0,10 g
BO_3H_3	0,05 g
SO_4H_2 66°B	1 ml

Pour the different products in water in the above indicated order and agitate each time.

Agitate energetically after the final addition of sulfuric acid (1 ml.).

Wait 15 minutes.
Filter on paper.
Divide into 5 ml. ampules and flame-seal them.
Autoclave 30 minutes at 110° C.

Preserve in the freezer at 4° C.
Duration of preservation: one year or more.
(1.6 ml. for 1 liter of eggs or 0.1 ml. for 100 ml. yield or medium).

Other oligoelements -- Outside of those contained in ossein, those contained in very finely sieved anthracite ashes, sterilized in a Pasteur oven (0.16 g. per liter of eggs or 0.01 g. for a 100 ml. yield of medium).

4% ossein for base medium
(to be prepared in advance)

Pour 960 ml. of distilled water in a large receptacle with a large opening. Heat in a water bath at 100° C.

Gradually add 40 g. of ossein (crude gelatin) while stirring with a glass rod.

After it is completely liquefied, bring the pH to 6.8 with the help of a 6% caustic soda solution, in the presence of an indicator.

Filter in a heated state, under a vacuum, on paper and in a Buchner filter.

Still in a heated state, divide the ossein in Pyrex balloon flasks, with 80 and 160 ml. per flask.

Autoclave for 30 minutes at 110° C, raising the temperature very slowly after the liquefication of ossein in a 37° C oven for one hour.

After autoclaving, close with a rubber stopper to avoid evaporation and store in rkness.

Always verify the appearance before using: clear yellow color, transparent.

20% ossein for BO medium: "sous cape" culture.
(Base medium + exuded ossein).

Distilled water	800 ml.
Ossein (crude gelatin)	200 g.

Same preparation as for 4% ossein.

After neutralization to pH 6.8 and autoclaving 30 minutes at 110° C, divide in Pyrex balloon flasks with 80 and 160 ml. per flask.

Monkey organ pulp. Liver.
(For "double layer" culture)

A young cynocephalus monkey, free from all infirmities and parasites and having never served for other experiments, after general anesthesia with chloroform, is bled white by cardiac puncture and section of the two carotid arteries.

The liver is removed after laparotomy performed with extreme precautions for surgical asepsis.

The bile vesicle and the hepatic canals are fully excised and eliminated.

The liver is immersed in tepid sterile distilled water. It is then cut into small pieces and weighed.

After rinsing in sterile distilled water to eliminate the lacquered blood, the pieces of liver are minced with scissors and reduced to pulp.

Add three times its weight of bidistilled sterile water (300 ml. of water for 100 g. of liver).

Filter through two thicknesses of gauze.

Distribute in tinted ampules.

Then seal.

Tyndallize in the oven at 54-56° C.

Make sterility test.

Preserve in the freezer, at 4° C. Delay of utilization: six months. For kidneys, same preparation processes. For the brain, grind and add six times its weight of sterile bidistilled water. The extracts of lyophilized organs are slightly less active than pulp preserved in the freezer, at 4°.

MEDIA PREPARATION METHOD.

I. -- In a Pyrex balloon flask, having a 3 liter capacity, containing glass balls and 16 g. of potato-starch previously autoclaved 30 minutes at 110° C, add the solution of salts prepared in advance.

The flask is placed in a boiling water bath and is agitated constantly until complete homogenization (about 15 minutes). The

temperature of the water bath is then lowered to 56° C and maintained for one hour.

II. -- A few minutes before taking the flask from the water bath, add 0.16 g. of finely sieved sterile anthracite ashes and 1.6 g. of A. Berthelot and G. Bertrand's oligodynamic solution to the potato-starch -salts mixture. Agitate energetically and leave for a few minutes at 56° C (the rosy tint of the mixture will intensify).

III. -- Fresh eggs (from a supervised farm) with white and dark shells, are previously brushed in running water and immersed in non-denatured 90° alcohol for one hour and are then broken one by one and poured into a graduated cylinder.

Measure 800 ml. of whole eggs and 200 ml. of yolks alone. These eggs are beaten and homogenized for 10 minutes with the help of a glass rod.

IV. -- Pour the homogenized eggs into the solution of salts taken from the water bath 15 minutes earlier to avoid the formation of clots from contact with a too hot solution (the mixture is the color of caramel cream).

V. -- Filter through gauze (three thicknesses) with all sterile precautions.

Before passing to the following steps which constitute the final operations in the medium preparation, a triple choice is offered:

a) Either prepare all as base medium (B);

b) Either prepare all as BO medium (base + exuded ossein);

c) Either, as we recommend, prepare half as base medium (B) and half as BO medium.

First choice:

a) Total volume made into base medium (B).

For this:

1. In a balloon flask containing 160 ml. of 4% ossein, sterile and at pH 6.8, previously liquefied by water bath heating or by a 30 minute stay in a 37° C oven, pour 16 m. of 2% malachite green and agitate energetically.

2. Pour the 4% ossein mixture + malachite green in the flask containing the eggs and the solution of salts and agitate just until the medium has an homogenous color.

3. Place the terminated medium in the oven at 37° C. for an hour before proceeding to the division into 170 x 17 mm. notched tubes.

Second choice:

b) Total volume made into BO medium (base + 20% exuded ossein).

For this:

Add 16 ml. of 2% malachite green to a balloon flask containing 160 ml. of 20% sterile ossein having a 6.8 pH (previously liquefied).

Then proceed exactly as for the base medium.

Third choice:

c) Half of the volume made into base medium (B); half made into BO medium.

*.... 8 ml. of 2% malachite green and 80 ml. of 4% ossein. Stir energetically and place the flask in the oven at 37° C. for an hour.

In balloon flask II (BO media for the "sous cape" culture).

To 710 ml. of media add a mixture of 8 ml. of 2% malachite green and 80 ml. of 20% ossein. Agitate energetically and place in the oven at 37° for an hour.

DISTRIBUTION

Distribute the base medium in flask I and the BO medium in flask II separately and successively.

The medium, after a one hour stay in the 37° oven will be agitated energetically and then distributed into sterile 170 x 17 mm. tubes, notched at 6 cm. from the neck, with 6.5 ml. per tube.

It is important to agitate the flask from time to time during distribution to avoid sedimentation of the medium.

Coagulation will be produced by humid heat (coagulator saturated with purified water vapor) in a single session, at 85° for 45 minutes.

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*Translator's note: The last four lines of page 486 were covered by another page when the xerox was made.

The media will be coagulated in a strictly horizontal position, the 5 mm deep notch makes this operation very easy. Surface offered for culture, 15 cm^2.

OSSEIN EXUDATION OF THE BO MEDIUM.

Only the BO medium, having received 20% ossein will exude a portion of this ossein at the lower portion of the medium. To obtain this exudation, operate as follows:

As soon as they leave the coagulator, the tubes containing BO medium are arranged in a strictly vertical position very close to each other, in a box with several racks and placed in a warm room or in a 30° oven for about 12 hours.

This finished, the tubes are capped. A 0.3 ml. gel occupies the bottom of the tubes which will be preserved in the freezer at 4° while the tubes of base medium (B) can be stored anywhere after coagulation.

We believe that all the egg media should be preserved shielded from daylight because a few hours exposure lower their sensitivity to 50%.

For media which must be inoculated within eight days after preparation, preservation in the 4° freezer has no advantage.

RESPECTIVE INSTRUCTIONS FOR THE MEDIA AND CULTURAL METHODS

In a very schematic manner the respective instructions for the media and the cultural methods suggested are the following:

1. For bacilli called or thought to be normal, coming from respiratory tracts (expectoration, gastric tube, bronchio-aspiration, laryngo-tracheal samples) and in a general fashion for all the bacilli in active multiplication, the best results -- in number, rapidity of appearance and luxuriance of colonies -- are obtained on the base media (B) by surface culture in the usual manner.

2. For bacilli having an extra-pulmonary origin (urine, L.C.-R., S.F. discharges and biopsy samples) the best results are obtained by "sous cape" culture of BO medium (base medium + 20% exuded ossein on the lower portion of the medium). By this method the bacilli are covered by a thin pellicle of ossein which protects them from contact with atmospheric air during the initial phase of culture while allowing them to have access to free air after a certain number of cellular divisions.

This cultural method facilitates the in vitro development of "quiescent" bacilli and the bovis variety of M.t., which as we know, preferably causes extra-pulmonary lesions.

3. "Nutritive double layer" culture, and especially culture in the presence of organic extracts coming from the monkey, seems to be indicated and more effective when the bacilli are suffering and incapable of pronounced in vitro biosynthesis.

This cultural method is produced by introducing V drops of monkey organ pulp, prepared and preserved as above, into tubes of base medium (B) or BO, just before inoculation.

INOCULATION TECHNIQUE

Of all the precautions given (4), the most important are:

1. Stop antibiotic therapy the days which precede the sampling.

2. Use fresh samples.

3. Never use 9 per 1000 salt water, or physiological water, in the manipulations.

4. Avoid rapid and extensive centrifugations.

5. Avoid homogenization purification for sterile samples (L.C.-R., sterile S.F. liquides, etc.).

6. Treat the infected products for the minimum of time with the weakest concentration of the homogenizing substance.

7. Place the media in the 37° oven for 30 minutes in a vertical position before inoculation.

8. Make sure the culture media are never exposed to sunlight.

9. Use enough tubes: four tubes for each variant which will be inoculated, preferably with a graduated pipette, with 0.25 ml. per tube (see note).

10. Cap the tubes immediately, the residual humidity being favorable for the culture starting, and place them in the oven in a horizontal position aligned either on a rack provided for notched tubes, or on a plateau provided with a full glass rod placed under the notch of the tubes.

Note: If BO medium (base medium + ossein conveyed and exuded by the medium) is not used, it is still very easy to have a "sous cape"

culture. For this, to several tubes of coagulated base medium add V drops of 4% previously liquefied ossein, in a sterile manner and before inoculation.

Note that along with monkey organic extracts, all types of associations with vitamins or other substances can be produced extemporaneously.

RESULTS

They are a function of the origin, the nature and the physiopathological conditions of the bacilli in the sample.

Colony appearance. -- In comparison with Jensen's medium, the colonies are more humid and their morphology varies according to the sample: spread, raised, crown shaped or depressed in the water (sheet I, Fig. 1 and 2).

Precocity. -- In relation to Jensen, they are all the more precocious as the bacilli which give rise to them are more disturbed in their metabolism. The advance is not very significant for normal vitality bacilli but is on the order of one to several weeks for lacking and dysogonic bacilli.

Luxuriance. -- No matter what nature and type of bacilli are used, the size of the colonies obtained is always greater than on Jensen's medium. It varies from 1 to 10 mm in diameter and the weight of 5 mg. per colony is rapidly reached and passed.

Number. -- It constitutes the most important element to compare the sensitivity of the two media. Here again the difference is all the larger between our media and Jensen's medium as the cultural difficulties on the latter are more pronounced.

1. For bacilli coming from respiratory tracts in free communication with the bronchials, not attacked by antibiotics and having a normal vitality, there is little or no difference between Jensen's medium and the base medium, both assuring a surface culture.

On the other hand, and then only for rapidly multiplying bacilli, the colonies are definitely fewer on the BO medium ("sous cape" culture) and on media enriched with organ extracts ("double layer culture").

This is due to the fact that the normal bacilli in active multiplication need more air than supplementary stimulating substances.

2. For bacilli of extra-pulmonary origin and bovine type bacilli, the number of colonies is sometimes equal on Jensen's medium and the base medium, but the appearance of macroscopic colonies on the base

medium and BO ("sous cape" culture) is always one to several weeks earlier. Besides, the size of the colonies is two to four times greater (sheet II, Fig. 3 and 4).

3. For the bacilli in a "state of quiescence," for those attacked by antibiotics and a fortiori for bacilli deeply disturbed in the metabolism -- bacilli called "visible and non-viable" -- the cultural differences are such that there is no question of establishing a parallel (sheet III, Fig. 5 and 6, sheet IV, Fig. 7 and 8).

Actually culture on our media, and in particular the "sous cape" and the "double layer" cultures can yield a surface colony one to several weeks earlier and give a number of colonies two to ten times higher. In some cases the culture is positive, while it was uncertain (non-transferable colonies) or completely negative on Jensen's medium.

In conclusion: the viability and vitality "potential" of bacilli coming directly from the organism being unknown and unforeseen, the heterogenicity and the heterotrophy for all bacillary populations attacked by antibiotics being the rule, the single culture method can no longer be considered as sufficient.

Also taking into account the very particular requirements of bacilli disturbed in their metabolism, the use of even richer media than the usual media and varied cultural methods (surface, "sous cape" and "double layer") are necessary.

In proceeding thus, even with bacilli called "visible and non-viable," the cultural failures will become completely exceptional.

DISCUSSION

From our personal verifications, one fact stands out: among the bacilli reputed to be non-viable which are reanimated by "sous cape" and "double layer" culture, all types, from sensitive to poly-resistant can be represented.

The qualitative and quantitative study of the sensitivity to antibiotics and the total or individual catalistic activity of the colonies obtained from bacilli apparently non-viable, testifies to the diversity and the heterogenicity of these strains.

The hypothesis according to which the bacilli reputed to be "visible and non-viable" would be a priori and almost necessarily INH-resistants, is not confirmed by these facts.

However, taking into account that resistance to INH is very frequent, it is completely normal that among the "reanimated" strains we would meet more INH resistants.

On the other hand, it appeared to us that among bacilli which are difficult or impossible to cultivate on usual media, a number were stimulated by very weak titers (0.05 - 0.1 µg) of PAS (para-aminosalicylic acid) (probable salicylic effect) (26, 27, 28).

Elsewhere, the revealing of quiescent and sensitive elements in a population, which is resistant in its immense majority, is not surprising. Likewise, the possibility of a possible selection by means of transplanting or passage to a guinea pig is not to be set aside, the most vigorous elements having precedence over the others.

As for us, we believe that the reanimated bacilli are in the image of what they were when they entered quiescence.

In spite of appearances, it would be inexact to say that there is the possibility of a mutation of the avirulent towards the virulent, by means of particular cultural conditions.

Clinical evolutions occurring in patients eliminating non-viable bacilli which we had "reanimated" a few weeks or months before:

a) attest to the permanence of virulent bacillary units or groupings in the heart of populations reputed to be avirulent or non-viable;

b) confirm the advantage of varied cultural methods to uncover these bacilli;

c) invalidate the little likely hypothesis of a mutation of avirulent bacilli to virulent bacilli.

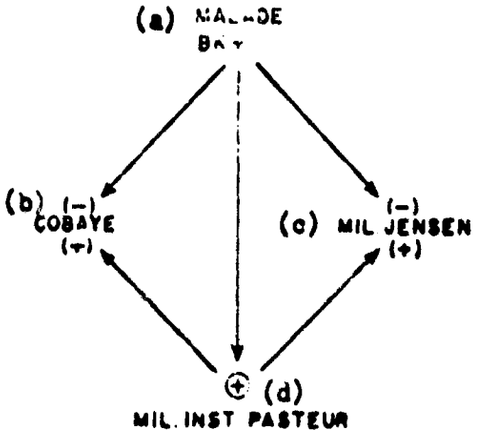

Fig. 1.

Legend: a - Patient BK; b - Guinea pig; c - Jensen's medium; d - Institut Pasteur Medium

With this goal and to remove all hypothetical fear of a return to virulence by the avirulent strains -- by means of our media -- we performed the following experiment:

Guinea pigs were inoculated with BCG (bacillus Calmette-Guerin) from Jensen's medium and with BCG which had been passed several times on our medium.

No difference between the two lots of guinea pigs was noticed, of brief or long duration.

What we can advance with certitude is that in many cases our media constituted a "relay" between the human organism and the guinea pig organism on one hand and between the bacillus laden pathological product and the usual media on the other hand. This can be schematized as found in Figure 1.

However, it happens that inoculation of the organs of guinea pig, which have been tuberculized by bacilli reanimated on our media, into Jensen's media fails partially or totally and that "sous cape" or "double layer" culture will again be necessary to recover the strain.

SUMMARY

In the light of our investigations, it appears that the bacilli described as "visible and non-viable" are actually bacilli of reduced vitality, of ephemeral viability or "in a state of quiescence."

Often enough surface culturing on the usual media is insufficient, being incapable of ensuring their development.

To remedy this, we have prepared an egg-medium enriched with oligo-elements, pyruvate and glutamate of sodium, and ossein.

This basic medium which is more sensitive than the Lowenstein-Jensen medium, can be used for three methods of culture:

1. Surface culture (basic medium) such as usually practiced, assuring the development of bacilli of normal or only slightly reduced vitality.

2. "Sous cape" culture (basic medium + exuded ossein = BO):

a) Protects the bacilli from direct contact with air in the initial stage,

b) Accelerates the growth of bacilli of extra-pulmonary origin,

c) Reveals bacilli which are in a state of quiescence.

3. "Double layer" culture (basic medium or BO medium + extracts of monkey organs added to the surface);

a) Favors the growth of bacilli with greatly disturbed metabolism,

b) Stimulates the multiplication of "quiescent bacilli,"

c) Ensures the reanimation of bacilli hardly able to realize biosynthesis in vitro and in vivo (in guinea pigs) and for this reason considered as non-viable until today.

The demonstration in vitro of quiescent bacilli, the reanimation of bacilli considered until now as non or only slightly viable, the confirmation of their pathogenicity for guinea pig , throw new light on certain problems of a pathogenic and epidemiological nature which had not previously been solved.

BIBLIOGRAPHY

1. Bernard (E.), Lotte (Mlle A.) and Pretet (S.), Bull. Acad. Med., 1959, 143, 462.

2. Jenson (K. A.), Bull. Union intern. Tub., 1952, 22, 16.

3. Coletsos (P. J.), Oriot (E.), Regel (N. de) and Machavoine (M. G.), Bull. Acad. Med., 1959, 143, 479.

4. Coletsos (P. J.), Oriot (E.), Regel (N. de) and Machavoine (M. G.), Ann. Inst. Pasteur, 1959, 97, 135.

5. Coletsos (P. J.), Oriot (E.), Regel (N. de) and Machavoine (M. G.), Ann. Inst. Pasteur, 1959, 97, 210.

6. Dubos (R. J.) and Davis (B. D.), J. exp. Med., 1946, 83, 409; 1947, 86, 215.

7. Dubos (R. J.), J. exp. Med., 1947, 85, 9; 1948, 88, 73.

8. Dubos (R. J.), Davis (B. D.), Middlebrook (G.) and Pierce (C.), Am. Rev. Tub., 1946, 54, 204.

9. Youmans (G. P.) and Karlson (A. G.), Am. Rev. Tub., 1947, 55, 528.

10. Dubos (R. J.) and Middlebrook (G.), Am. Rev. Tub., 1947, 56, 334. J. exp. Med., 1948, 88, 61.

11. Dubos (R. J.) and Noufflard (Mlle H.), Ann. Inst. Pasteur, 1950, 78, 208.

12. Melvin (I.), Klein (G. C.), Jones (N.) and Cummings (M. M.), Am. Rev. Tub., 1951, 63, 459.

13. Calmels (J. R.) and Boisvert (H.), Ann. Inst. Pasteur, 1952, 83, 338.

14. Berte (S. J.) and Tucker (E. B.), Am. Rev. Tub., 1960, 81, 595.

15. Dorset, J. Am. Med. Assoc., 1902, 555.

16. Lowenstein, Deut. Med. Wschr., 1930, 1010. Zbl. Bakt., 1931, 120, 127.

17. Herold (R. D.), J. inf. Dis., 1931, 48, 236.

18. Petragnani, Boll. Inst. sier. milan., 1926, No. 3, 173.

19. American Trudeau Society (A.T.S.), Am. Rev. Tub., 1946, 54, 428.

20. Jensen (K. A.), Zbl. Bakt., 1932, 125, 222. Acta Tub. Scand., 1940, 14, 125. Bull. Union intern. Tub., 1954, 24, 81.

21. Coletsos (P. J.), C. R. Conf. intern. Madrid, 1954, 374.

22. Middlebrook (G.) and Cohn (M. L.), Am. J. publ. Hlth, 1958, 48, 844.

23. Ficher (M. W.), Am. Rev. Tub., 1954, 69, 797.

24. Coletsos (P. J.), C. R. Acad. Sci., 1959, 248, 3497.

25. Berthelot (A.), Bull. Soc. Chim. biol., 1934, 16, 1553.

26. Bernheim (F.), J. Bact., 1941, 41, 387.

27. Lehmann (J.), Lancet, 1946, 1, 15.

28. Bonicke (R.), Bull. Union intern. Tub., 1957, 27, 151.

Sheet I

Usual macroscopic appearance of bacillary colonies.

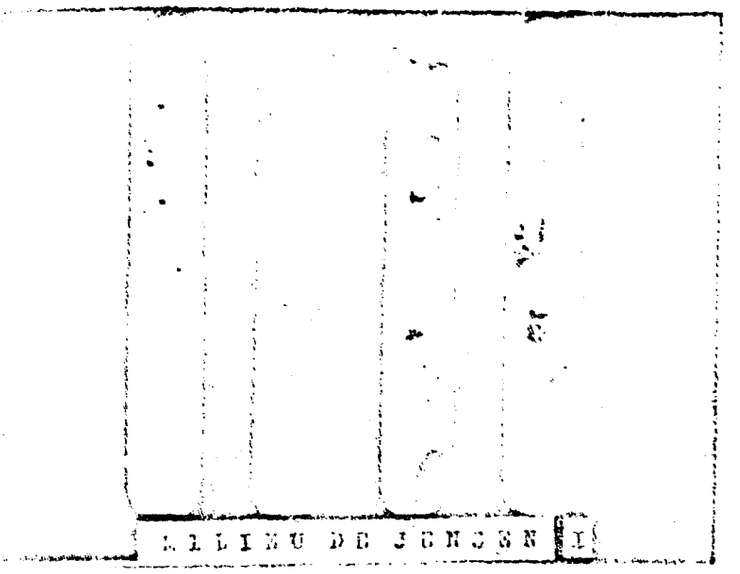

Fig. 1 -- Jensen's medium.

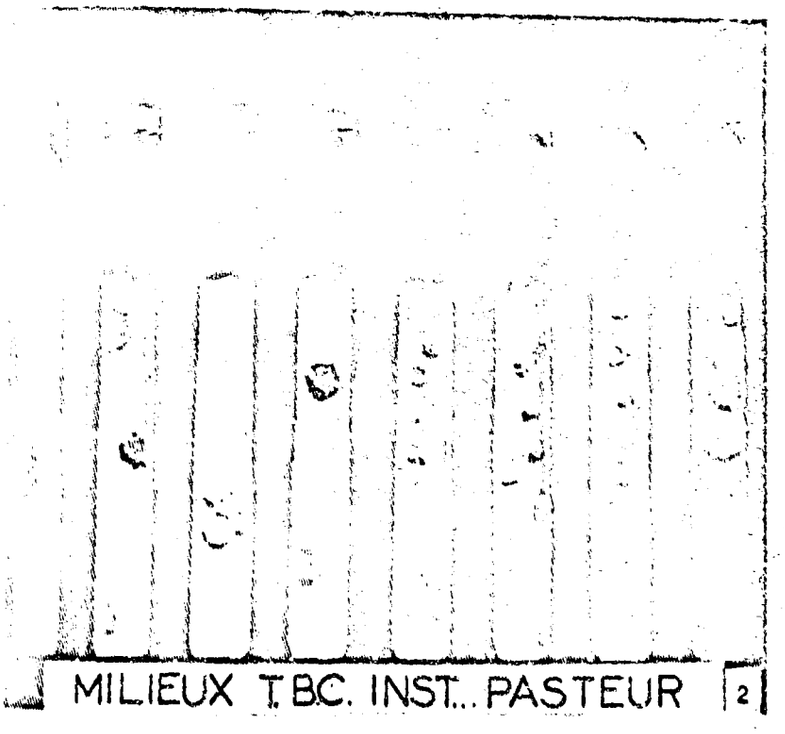

Fig. 2 -- On Institut Pasteur TBC medium.

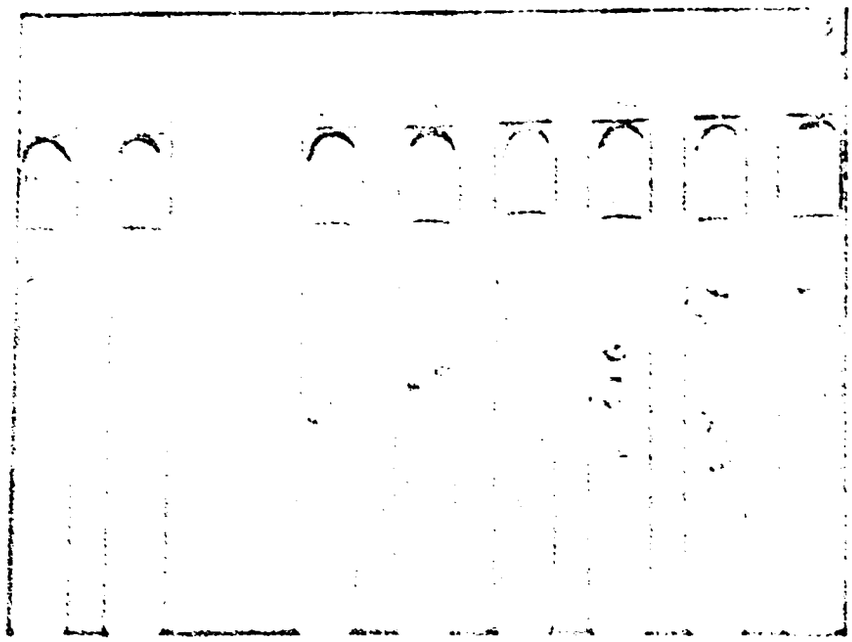

Fig. 3. Mr. N... Surgical sample (tuberculosis f the wrist). From left to right: Jensen's medium, 2 tubes: negative culture: base medium (surface culture), 2 tubes: 8 colonies; BO medium ("sous cape"), 2 tubes: 16 colonies; BFS medium (nutritive double layer culture), 2 tubes: 60 colonies.

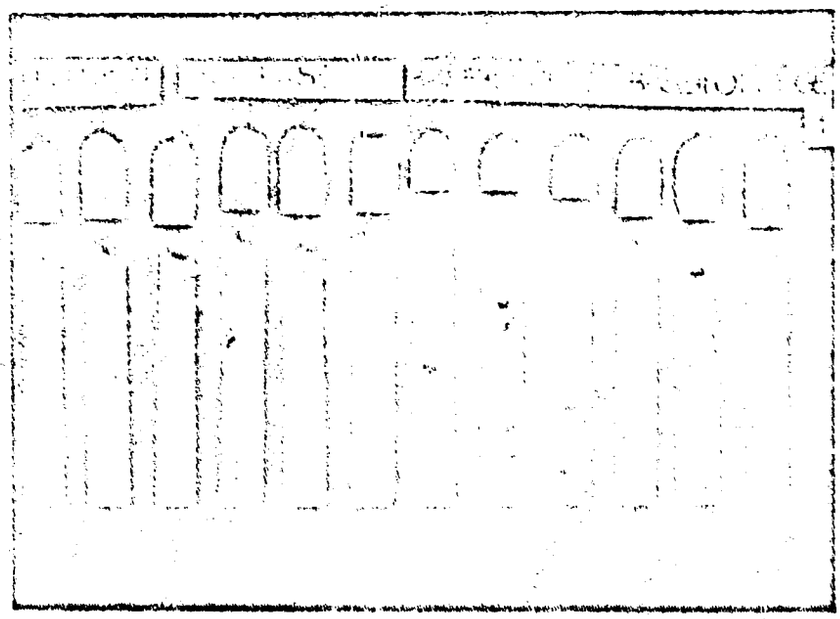

Fig. 4. Child L... L.C.-R. From left to right: Jensen's medium, 3 tubes: negative culture; base medium (surface culture), 3 tubes: one colony; BO medium ("sous cape"), 3 tubes; 9 colonies; BFS medium (nutritive double layer culture), 3 tubes; one colony.

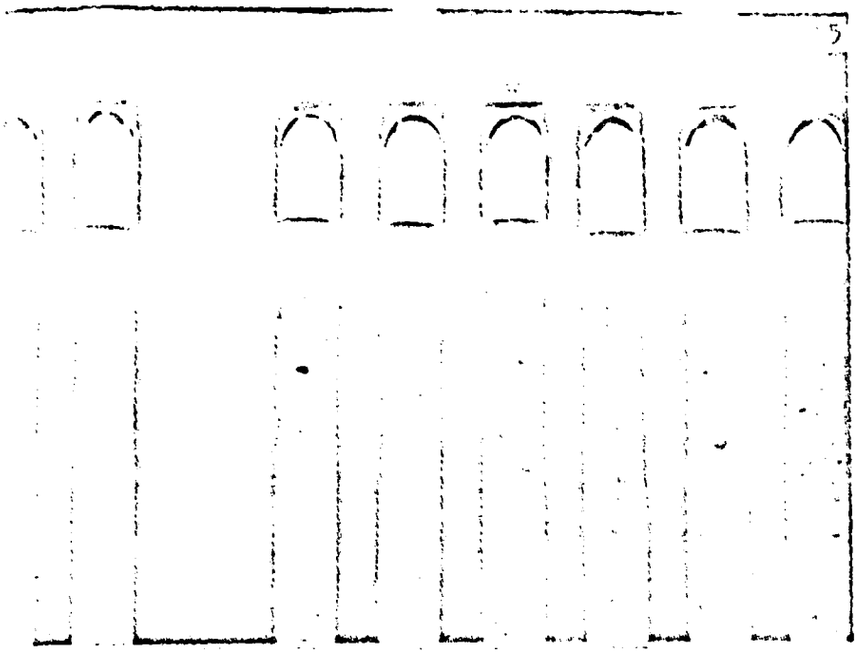

Fig. 5. Mrs. M... Expectoration (microscopic examination: 2 bacilli per field). From left to right: Jensen's medium, 2 tubes: rare colonies at the limit of visibility, non transferable; base medium (surface culture), 2 tubes: 30 colonies of which 15 are eugonic; BO medium ("sous cape" culture), 2 tubes: 40 colonies, of which 25 are eugonic; BFS medium (nutritive double layer culture), 2 tubes: 50 to 60 colonies per tube of which 50% are eugonic.

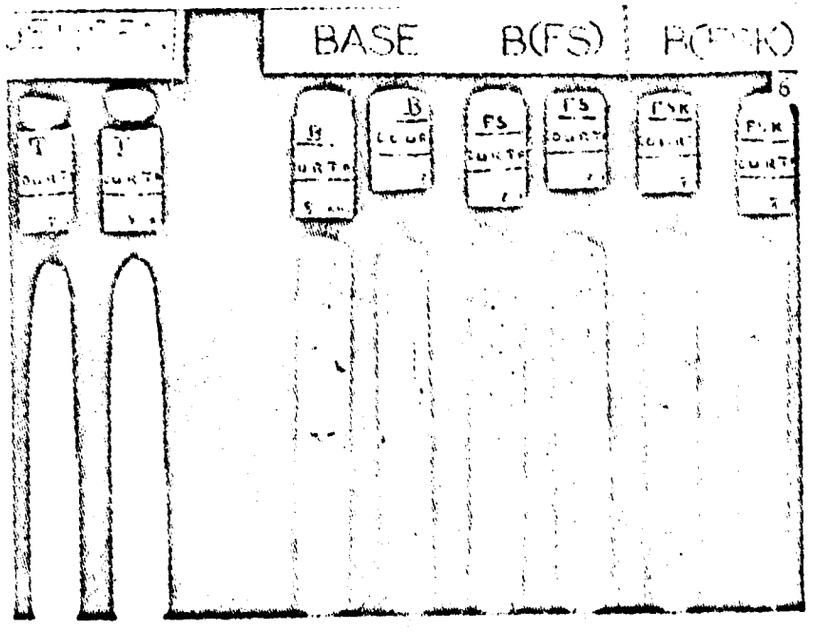

Fig. 6. Mr. C... Urine (chronic renal tuberculosis). From left to right: Jensen's medium, 2 tubes: one colony; base medium (surface culture), 2 tubes: 5 colonies: BFS medium (nutritive double layer culture), 4 tubes: 80 to 100 colonies per tube.

Fig. 7. Mrs. V., Expectoration (macroscopic examination: 2 to 4 bacilli per field). From left to right: Jensen's medium, 2 tubes; 2 colonies at the limit of visibility; base medium (surface culture), 2 tubes; 3 giant colonies; BO medium ("sous cape" culture), 2 tubes; 10 large colonies; BFS medium (nutritive double layer culture): 12 large colonies plus 20 small ones.

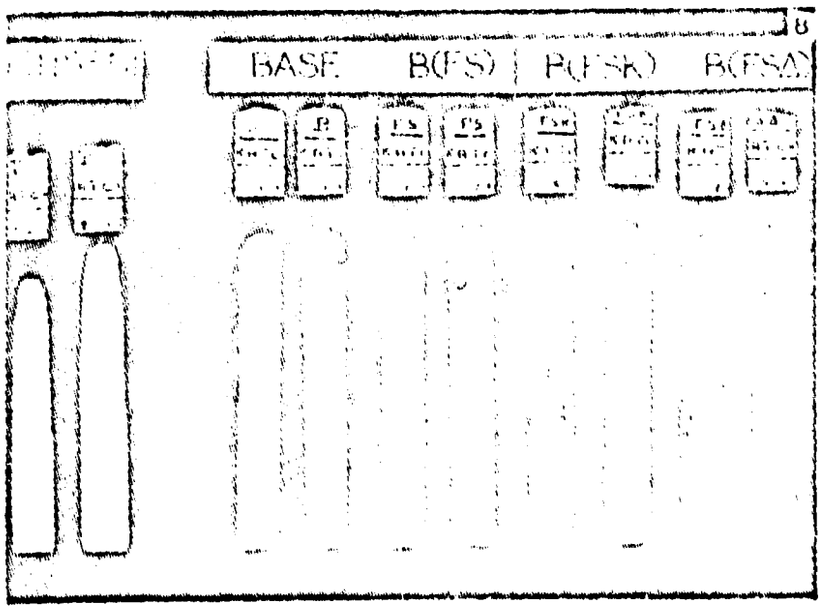

Fig. 8. Mr. K... Expectoration (microscopic examination: 50 bacilli per field). 1. Culture on Jensen's: negative several times; 2. On base medium (surface culture): one colony; 3. Culture in the presence of organic extracts: 2 colonies; 4. Organic extracts + Vit. K: 150 to 200 colonies per tube; 5. Organic extracts + traces of P.A.B.: 4 colonies per tube.